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Seeing the Light: Lasers, Fluorochromes, and Filters

Because flow cytometry involves the illumination of particles by a light source and the subsequent analysis of the light emitted by particles after this illumination, an understanding of some of the principles behind light production, light absorption, and light emission is important for the effective design and interpretation of experimental protocols. The principles of photochemistry apply both to the generation of light by a light source and to the absorption and emission of light by a fluorochrome. It is best to get these concepts straight before we begin to describe the staining of cells.

GENERAL THEORY

We need to begin with a brief review of atomic structure. Atoms consist of relatively compact nuclei containing protons and neutrons. At some distance from these dense nuclei each atom has electrons moving in a cloud around the central nucleus. The electrons move in *shells* or *orbitals* or *probability waves* (different words derived from more or less classic or quantum mechanical terms of reference) around the nucleus, and the number of electrons circulating in these orbitals depends on the element in question. Four things are particularly important for flow cytometrists to understand about these electrons: First, atoms have precisely defined orbitals in which electrons may reside. Second, an electron can reside in any one of the defined orbitals but cannot reside in a region that falls between defined orbitals. Third, the energy of an electron is related to the orbital in

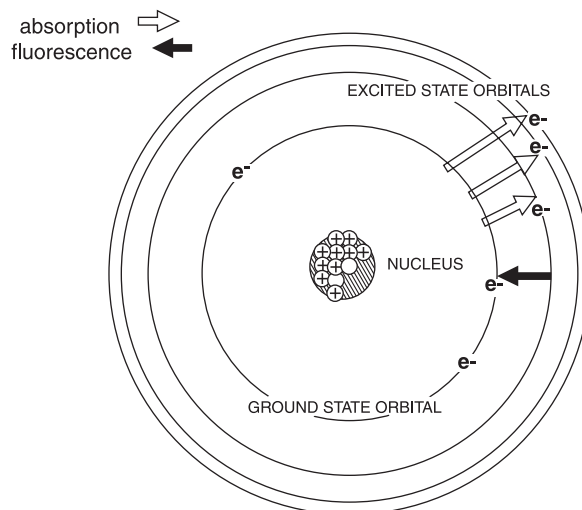


Fig. 5.1. An “old-fashioned” but conceptually easy diagram of an atom with electrons circling the nucleus. Electrons can absorb energy to raise them to an excited state orbital. When they fall back to their ground state, they may emit light, which we call *fluorescence*.

which it resides at any given moment. Fourth, an electron can absorb energy and be pushed to an excited (higher energy) orbital, but it will quickly give back that energy as it rapidly returns to its stable, ground-state configuration (Fig. 5.1). The energy given back as an electron returns from an excited orbital to its ground-state orbital can be in the form of light.

With my apologies to all physical chemists for simplifying electronic structure to four facts, we can now add to our knowledge one more fact about light itself. Light is a form of energy made up of photons, and the color of the light is related to the amount of energy in the photons of that light. For example, when red light is emitted by an object, that object is releasing photons of relatively low energy; blue light, in contrast, is made up of photons of higher energy. *Wavelength* (expressed in nanometers, abbreviated to nm) is a term often used to describe the color of light. The wavelength is inversely related to the amount of energy in the photons of that light: Blue light has wavelengths in the range of 400–500 nm, and its photons have relatively large amounts of energy; red light has wavelengths

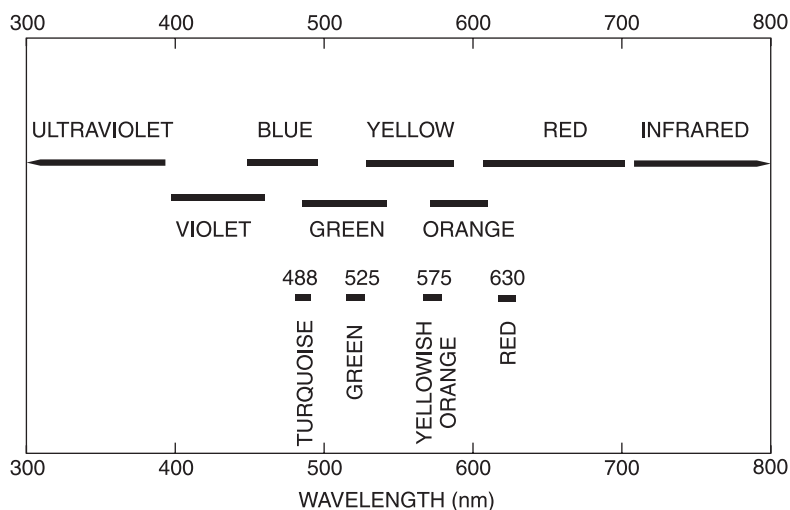


Fig. 5.2. Light, in the region to which our eyes respond, can be described by a “color” or, more precisely, by a wavelength. Photons with longer wavelengths have less energy.

of about 600–650 nm and photons with less energy than those in blue light. The other colors of the visible spectrum fall between these values (Fig. 5.2). Infrared light has a longer wavelength than red light (and less energetic photons); ultraviolet light has a shorter wavelength than blue light (and more energy). With this information, we can start to apply our knowledge of light and atomic structure to flow cytometry.

LASERS

The illumination of particles as they flow past a light source is responsible for generation of the signals upon which flow cytometric analysis is based. The illumination of particles can be provided by an arc lamp or by a laser. In either case, electrons within the light source are raised to high-energy orbitals by the use of electricity, and energy is given off in the form of photons of light when the electrons fall back to their lower energy orbitals. The color of that light is determined by the energy differences between the excited and lower orbi-

THE BASIC ION LASER

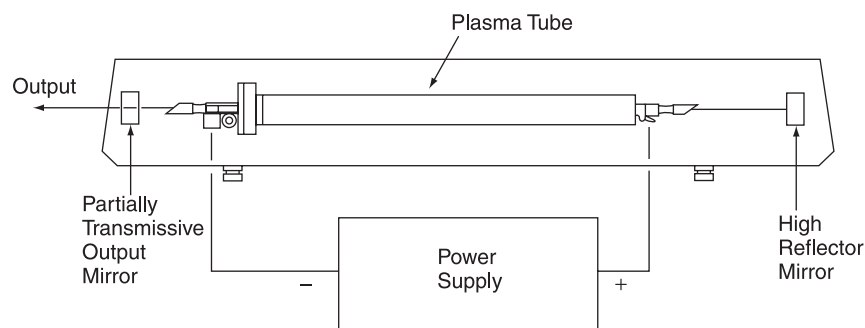


Fig. 5.3. The structure of a basic gas ion laser. Reproduced with permission of Spectra Physics.

tals of the atoms in the lamp or laser. Lasers and arc lamps each have certain advantages. Because lasers are the light source of choice in most current flow systems, it is important to understand the definite restrictions on experimental flexibility that are imposed by the way in which a laser works.

Gas lasers consist of tubes (called *plasma tubes*) filled with gas; a cathode lies at one end and an anode at the other (Fig. 5.3). A voltage is applied across the plasma tube in order to raise the electrons in the atoms of gas to excited orbitals. As the electrons fall back to lower energy ground states, they give off energy in the form of photons of light; the color of the emitted light is determined by the type of gas used and is a function of the differences between the energy levels of its atomic orbitals. Reflecting mirrors are placed outside the tube at either end. If the reflecting mirrors are aligned precisely with the plasma tube, then the photons given off by the gas will be reflected back and forth through the tube between the two reflecting mirrors. The applied voltage maintains the electrons in the gas in excited orbitals. The reflected photons, as they oscillate back and forth within the tube, interact with these excited gas ions to stimulate them to release more photons of identical energy (in a way predicted by Einstein). An amplification system thereby results: The photons oscillating back and forth between the two end mirrors cause more and more light to join the beam. By allowing a small percentage of

this oscillating beam to leave the system at the front mirror, we have generated what is known as a *coherent* light source.

A laser light source is coherent in three respects. It is coherent as regards its direction in that a beam is generated that diverges little and remains compact and bright for great distances (as in laser light shows and “star wars”). It is coherent with respect to polarization plane (of possible relevance for some specialized aspects of cytometry). Finally, it is coherent with respect to color because the electrons in a gas atom or ion are restricted in the orbitals that they use under plasma tube conditions and are therefore restricted in the amount of energy that is emitted when they fall from a higher energy to a lower energy state.

Coherence with respect to direction is the reason lasers are useful in flow cytometry: They provide a very bright, narrow beam of light allowing particles flowing in a stream to be illuminated strongly for a very short period of time so that measurable signals from one particle can be generated and then separated by darkness from signals generated by the following particle (refer back to Figs. 3.1 and 3.5). The disadvantage of this spatial coherence is that cells in a stream must be well aligned in the center of that stream if they are going to be uniformly illuminated.

Although spectral purity has certain advantages, coherence with respect to color is actually one of the major limitations of a laser system. Whereas an ordinary light bulb will put out light of a wide range of colors (a white light bulb puts out a mixture of the whole range of colors in the visible spectrum), the color of the output from a laser is restricted by the restricted range of electron orbitals that will support lasing in any particular gas. Argon ion lasers are the most common lasers used in flow cytometry today; they emit useful amounts of light at 488 nm (turquoise) and at 514 nm (green), as well as small amounts of ultraviolet light. By using a prism or a wavelength-selective mirror, the operator or manufacturer can choose one or the other of these colors, but there is relatively little light available in other regions of the spectrum (Fig. 5.4). Helium-neon lasers or red diode (solid-state) lasers are also used (often in conjunction with an argon ion laser in a dual laser benchtop flow system); a helium-neon laser puts out red light at 633 nm and a red diode laser at 635 nm. Research instruments may have three lasers, with one or two argon lasers for 488 nm and ultraviolet excitation plus a third HeNe laser

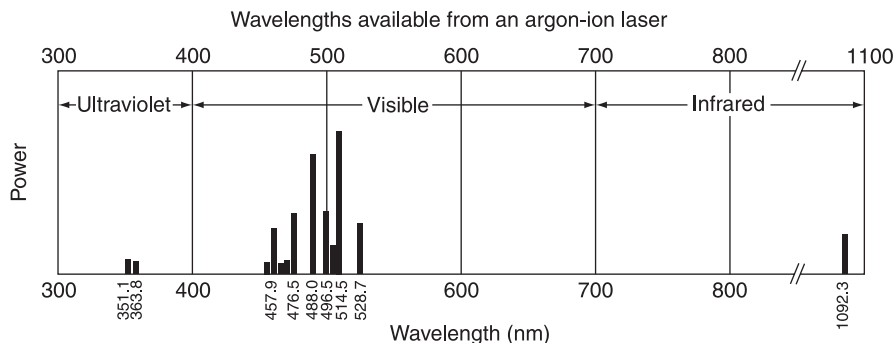


Fig. 5.4. The wavelengths of light emitted by an argon ion laser. The most powerful (and therefore most useful) wavelengths are 488 and 514 nm. High-power argon ion lasers can provide some useful light in the ultraviolet range.

or dye laser for red excitation. As a general principle, flow cytometers all have argon lasers emitting light at 488 nm; more advanced instruments will add additional lasers with additional wavelengths as required (Table 5.1).

A laser is therefore very good at providing bright, narrow, stable

TABLE 5.1. Laser Options with Examples of Some Possible Fluorochromes

Laser type	Wavelengths (nm)	Possible fluorochromes
Argon ion	488	Fluorescein, R-phycoerythrin, PerCP, PE-tandems, EGFP, EYFP, propidium iodide, Alexa 488, acridine orange
	514	Rhodamine, propidium iodide, EYFP, R-phycoerythrin
	Ultraviolet (351/364)	Hoechst dyes, DAPI, Indo-1, AMCA, Cascade Blue, EBFP
Helium-neon (HeNe)	Usually 633	Allophycocyanin, Cy5, TO-PRO-3
Krypton ion	568	Cy3, Texas Red, Alexa 568
	647	Allophycocyanin, Cy5, TOPRO-3
Red diode	635	Allophycocyanin, Cy5, TOPRO-3
Helium-cadmium (HeCd)	325	Indo-1, propidium iodide
	442	Mithramycin, chromomycin A3, ECFP

light beams of well-defined color and intensity. It is inflexible, however, in terms of the color of that light. In addition, large lasers are demanding in their requirement for routine, skilled maintenance; careful alignment between plasma tube, reflecting mirrors, output beam, and stream is essential. Furthermore, lasers are expensive; plasma tubes have limited lives and cost considerably more than an arc lamp when they need replacing. (It is worth noting that a well-aligned plasma tube with clean mirrors will last longer than a poorly aligned and dirty one because it will draw less current to produce the same amount of light; loving care of a laser can make a considerable difference to the overall running costs of a flow cytometer.) Many flow systems use small, air-cooled lasers. Air cooling is feasible when the laser is of low power and does not generate much heat (gas lasers are all relatively inefficient and generate a good deal of waste heat). However, cytometers with a stream-in-air analysis point have space and refractive index–mismatch surfaces between the cells and the light collecting lenses; as a result, detection of the light signals generated by the cells is inefficient. For this reason, sorting cytometers may require large, high-power lasers that put out very bright beams of light but also generate a great deal of waste heat. These sorting cytometers usually need a large supply of cold water to keep their lasers cool and also need skilled cleaning and alignment. The main fact to be kept in mind about lasers, as we move to a discussion of fluorochromes, is that, because lasers make use of electrons excited to a limited range of orbitals, they are restricted in the color of the light they emit.

FLUOROCHROMES

In a laser, electrons are excited by means of energy from an applied voltage. We then take advantage of the energy given off as light when the electrons fall back to lower energy states. With a fluorescent molecule (a “fluorochrome” or “fluorophore”), light itself (called the *excitation light*) is used to excite the electrons initially. We then analyze the emitted light (of a different color) that is given off as the electrons of the fluorochrome return to their ground-state orbitals. On the basis of our knowledge about electron orbitals together with our knowledge about laser output, it should now be apparent that, when a fluorochrome is illuminated, the electrons in that fluorochrome

can absorb energy if, and only if, the excitation light contains photons with just the right amount of energy to push an electron from one defined fluorochrome orbital to a higher (more energetic) defined orbital. Thus the difference between orbital energy levels of a fluorochrome will strictly determine what color of light that particular fluorochrome can absorb. When electrons fall back from their excited (more energetic) levels to their ground-state or less energetic orbitals, light is emitted of a color that depends on the difference in energy levels between the two fluorochrome orbitals in question. Because energy is not created and is, in fact, always lost to some extent as heat, the color of the light emitted when an electron falls back from an excited state to its ground state is always of a somewhat lower energy (that is, of a longer wavelength) than the energy absorbed in raising the electron to the higher orbital in the first place (Fig. 5.5). The light given off when an electron falls back from an excited state to its ground state orbital is called *fluorescence*. The time required for fluorescence to take place is approximately 10 ns after the initial activation of the fluorochrome by the excitation beam.

If we plot the colors of light that can be absorbed and the colors that are then emitted by various compounds (called *absorption* and

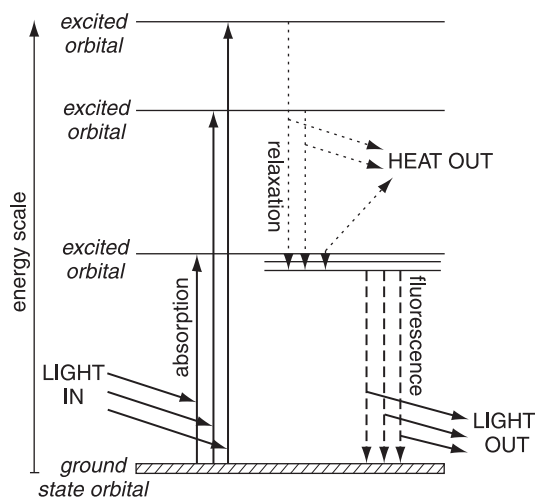


Fig. 5.5. The absorption of light by an electron and the subsequent emission of the energy from that light as both light and heat. Because some of the absorbed energy is lost as heat, the emitted light always has less energy (and is of longer wavelength) than the absorbed light.

emission spectra), we can see how these principles work in practice (Fig. 5.6). The wavelengths of light absorbed by a compound will depend exactly on the electronic orbitals of its constituent atoms; the light then given off as fluorescence is always of a longer wavelength than the absorbed light. The difference between the peak wavelength for absorption and the peak wavelength for fluorescence emission is known as the *Stokes shift*. Some compounds (for example, PerCP) have a larger Stokes shift than others (for example, fluorescein).

We are now in a position to understand why the use of a laser to provide the illuminating beam in a flow system restricts the choice of fluorochromes that can be used for staining cells. If we are using an argon ion laser with an output of light at 488 nm, we can consider as suitable stains those and only those fluorochromes that absorb light at 488 nm. Rhodamine, a stain used extensively by microscopists, absorbs light poorly at 488 nm and is therefore not useful in conjunction with a 488 nm laser. Stains like DAPI and Hoechst can be used with a high-energy argon ion laser tuned to its ultraviolet line, but cannot be used if the laser is tuned to 488 nm. Appropriate stains for 488 nm excitation include DiOC_n(3) for looking at membrane potential and propidium iodide and acridine orange for looking at nucleic acid content. With the 488 nm light from an argon laser, the situation is also ideal for staining cells with fluorescein (another standby of microscopists). In fact, this traditional allegiance to fluorescein (sometimes abbreviated as FITC; fluorescein isothiocyanate is the chemically active form of the dye that will conjugate to proteins) is the principal reason that argon ion lasers were initially selected for the first laser-based flow cytometers. Fluorescein absorbs light in the range of 460–510 nm and then fluoresces in the range of 510–560 nm, with a peak at about 530 nm (green); it can also be readily conjugated to antibodies, thereby providing specific fluorescent probes for cell antigens (Table 5.2).

Because multiple photodetectors are available, a flow cytometer has the ability to measure two or more fluorescence signals simultaneously from the same cell. To use several fluorochromes at the same time, cytometrists with only one laser required a group of stains, all of which absorb 488 nm light but which have different Stokes shifts so that they emit fluorescent light at different wavelengths and thereby can be distinguished from each other by the color of their fluorescence. Propidium iodide and fluorescein are a pair of fluorochromes that fulfill these criteria (having different Stokes shifts) and can be

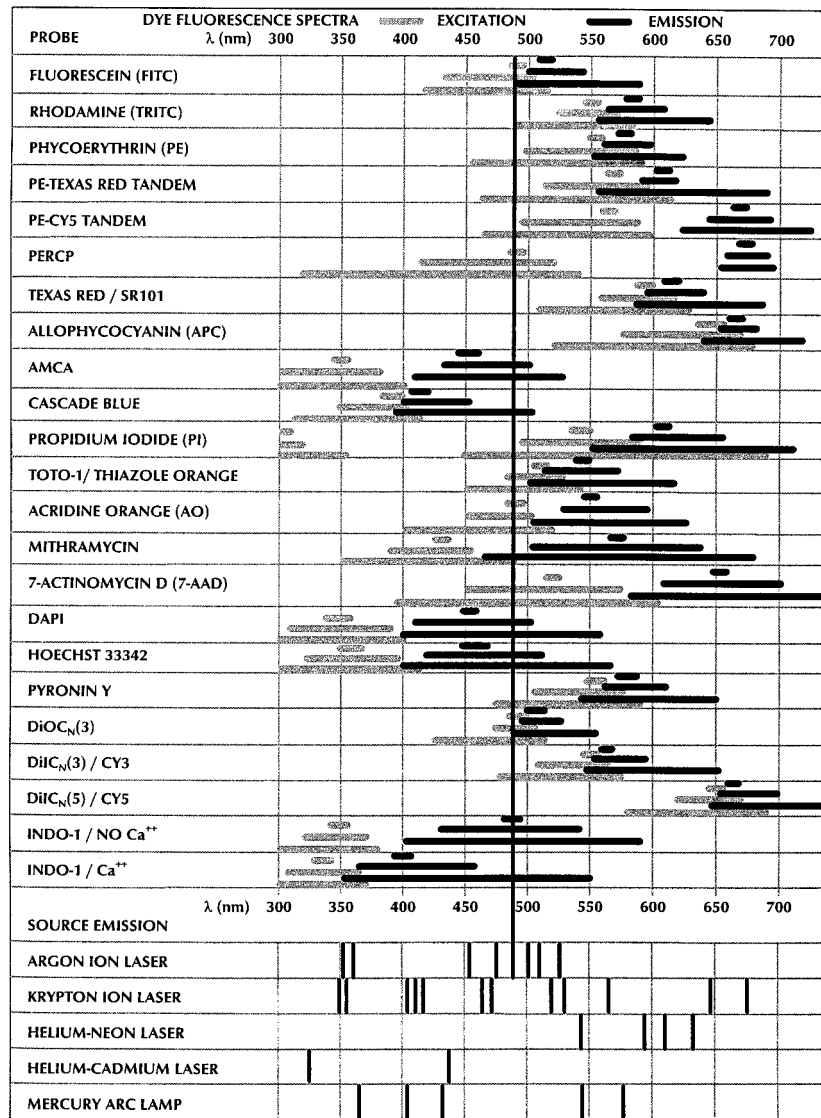


Fig. 5.6. The absorption (gray bands) and emission (black bands) spectra of various fluorochromes. The wavelength widths of the bands for each fluorochrome indicate the range of wavelengths that will be absorbed and emitted. Laser (excitation) wavelengths are indicated at the bottom of the chart. From Shapiro (1995).

TABLE 5.2. Absorption and Emission Wavelength Maxima of Some Useful Fluorochromes^a

Fluorochrome	Absorption (nm)	Emission (nm)
<u>Covalent labeling of proteins</u>		
Fluorescein (FITC)	492	520–530
R-phycoerythrin (PE)	480–565	575–585
PerCP	490	677
Alexa 488	494	519
PE–Texas Red tandem	480–565	620
PE–Cy5 tandem	480–565	670
PE–Cy7 tandem	480–565	755
Tetramethylrhodamine (TRITC)	540	570
Cy3	540	567
Alexa 568	578	603
Texas Red	595	620
Cy5	648	670
Allophycocyanin	650	660
<u>Nucleic acids</u>		
Hoechst 33342 or 33258	346	460
DAPI	359	461
Chromomycin A3	445	575
Propidium iodide	480–580 (also UV)	623
Acridine orange		
+ DNA	480	520
+ RNA	440–470	650
Thiazole orange	509	525
7-aminoactinomycin D (7-AAD)	555	655
TO-PRO 3	642	661
<u>Membrane potential</u>		
diOC _n (3) oxacarbocyanines	485	505
Rhodamine 123	511	546
<u>pH</u>		
Carboxyfluorescein		
High pH	495	520 (brt)
Low pH	450	520 (wk)
BCECF		
High pH	508	531 (brt)
Low pH	460–490	531 (wk)
<u>Calcium</u>		
Indo-1		
Low calcium	361	485
High calcium	330	405

(continued on next page)

TABLE 5.2 (continued)

Fluorochrome	Absorption (nm)	Emission (nm)
Fluo-3		
High calcium	490	530 (brt)
Low calcium	490	530 (wk)
<u>Reporter molecules</u>		
E (enhanced) GFP	489	508
EBFP	380	440
ECFP	434	477
EYFP	514	527
DsRed	558	583
FDG (β -galactosidase substrate)	490	525
<u>Cell tracking dyes</u>		
CFSE	495	519
PKH 67	485	500
PKH 26	510 and 551	567

^aThe absorption and emission maxima from this table will provide clues to the spectral ranges that are useful for excitation and for fluorescence detection with a particular fluorochrome. However, the absorption and emission spectra have breadth, with slopes and shoulders and secondary peaks (see Fig. 5.6). With efficient fluorochromes, excitation and fluorescence detection at wavelengths distant from the maxima may be possible. Therefore, inspection of the full, detailed spectra is necessary to get the full story. In addition, spectra may shift in different chemical environments (this will explain why maxima vary in different reference tables from different sources). Values in this table are derived primarily from the **Molecular Probes Handbook** and the article by Alan Waggoner (Chapter 12) in **Melamed et al.**

used together for analyzing nucleic acid content in fluorescein-labeled cells. Propidium iodide, however, is not fluorescent unless bound to double-stranded nucleic acid and therefore is not usefully conjugated to antibodies for the staining of cell surfaces. Flow cytometrists were therefore spurred to hunt for appropriate pigments (absorbing 488 nm light, fluorescing at a wavelength different from 530 nm, and capable of chemical conjugation to proteins). The humble seaweeds provided some of the answers.

Algae need to absorb light for photosynthetic metabolism, but many of them live in dimly lit regions beneath the surface of the ocean. In this inhospitable marine environment, they have evolved a rich assortment of pigments to help them capture light efficiently. Surveying this array of algal pigments, we can find compounds that absorb light and fluoresce at a range of different wavelengths. A quick inspection of the absorption and fluorescence emission spectra (Fig. 5.6) indicates that, with a light source of 488 nm, phycoerythrin

will absorb light fairly efficiently. With its longish Stokes shift, it fluoresces around 578 nm (orange). In addition, phycoerythrin was found to be almost as suitable as fluorescein for conjugating to antibodies or other proteins. For these reasons, fluorescein and phycoerythrin (PE) have become the fluorochromes of choice for dual color flow cytometry. They can both be conjugated to antibodies to provide specific reagents that fluoresce with different colors.

More recently, other algal pigments have been developed for flow cytometry. The dinoflagellate *Peridinium* has a photosynthetic apparatus that includes complexes of eight carotenoid (“peridinin”) molecules surrounding two chlorophyll molecules. This carotenoid:chlorophyll complex is called *peridinin chlorophyll protein* (marketed as PerCP by Becton Dickinson). The carotenoid molecules absorb blue-green light, and, because of the proximity of the chlorophyll molecules, the carotenoids will pass the absorbed energy (by a process called *nonradiative* or *resonance energy transfer*, i.e., no light is emitted) on to the adjacent chlorophylls. The chlorophylls, now with electrons in an excited state, can use this energy either for photosynthesis (in the alga) or for fluorescence (in the flow cytometer). In this way, the complex couples the absorption properties of one molecule with the emission properties of another molecule, shifting the wavelength of fluorescence from that of the primary fluorochrome to that of the secondary fluorochrome—thus effectively increasing the Stokes shift. PerCP can thus be used as a third color in conjunction with fluorescein and phycoerythrin.

In imitation of nature, organic chemists have engineered “tandem” fluorochromes that mimic the light-harvesting complexes of the algae. These synthetic pigments consist of two different fluorochromes bound together on a single backbone so that the first fluorochrome absorbs light from the laser and passes the energy on to the second fluorochrome, which emits the light at a relatively long wavelength. A common example of a synthetic tandem fluorochrome consists of a phycoerythrin molecule covalently linked to a Texas Red molecule or to a Cy5 molecule. In the presence of 488 nm light, the phycoerythrin moiety will absorb light, but, rather than fluorescing itself, will pass the absorbed energy on to the closely linked Texas Red or Cy5 molecule, which will then fluoresce at its own wavelength beyond 600 nm. These tandem molecules (whether natural or synthetic) provide an ideal way to increase the multicolor staining options when only a single 488 nm laser is available. The synthetic tandems, in particular,

do have certain intrinsic problems: The efficiency of light transfer between the primary and secondary fluorochromes of the tandem can vary depending on manufacturing procedures and on storage. This means that both the intensity and the color of its fluorescence can vary from one batch to another and over time. Although a common combination of fluorochromes for three-color analysis using 488 nm excitation is fluorescein, phycoerythrin, and a tandem of PE with Cy5, the use of these tandem fluorochromes can be avoided if more than one laser is available for excitation.

To increase options further within the limitations of the available fluorochromes, cytometrists are forced to purchase additional lasers. An additional laser will be focused to strike the stream at a secondary analysis point, usually downstream from the 488 nm illumination spot. By the use of a helium-neon or red diode laser, emitting light at 633–635 nm, fluorochromes such as allophycocyanin or Cy5 can be used. Allophycocyanin (APC) is a natural algal pigment that is a single molecule, not a tandem complex. Cy5 is a synthetic cyanine dye that can be modified to alter its absorption and emission characteristics (see Cy3 and Cy7). In addition, Molecular Probes (Eugene, OR) have developed an attractive set of fluorochromes (the Alexa dyes) that span the range of spectral properties suitable for various lasers. A cytometer with two argon ion lasers or with an argon ion and a helium-cadmium (He-Cd) laser can provide the option of using ultraviolet light at the same time as 488 nm light. This also increases the range of fluorochromes that can be analyzed. Research cytometers now are usually adapted for the implementation of two- or three-laser illumination. Increasingly, routine clinical cytometers have also begun to contain a second laser on their optical benches.

PARTITIONING THE SIGNAL WITH LENSES, FILTERS, AND MIRRORS

We have now described a system in which one or more narrow beams of laser light of well-defined wavelength are used to illuminate a cell, and the light scattered by the cell and emitted by various fluorochromes in or on that cell provide signals that are registered on a group of photodetectors. From our description of the optical bench in Chapter 3 (refer back to Fig. 3.7), we should recall that there are

three, four, or more detectors whose job it is to measure the intensity of the light coming out at right angles from the cells in the illuminating beam. The light coming out at right angles will be a mixture of colors, depending on the fluorochromes with which the cell has been stained. Because photodetectors are placed at different spatial positions in the cytometer, we need to use a combination of mirrors to direct light of a specific color toward its intended photodetector and not to any other (Fig. 5.7). Recalling that photodetectors are relatively color blind, it is these mirrors combined with colored filters that give each photodetector its own spectral specificity.

An optical mirror is vacuum coated precisely with thin layers of metal so that it will split a light beam by reflecting some of the light shining onto its surface while transmitting the rest. Mirrors that split a light beam according to color are called *dichroic mirrors*. A 640 nm short-pass dichroic mirror (640 SP), for example, if placed at a 45° angle in a light path, will transmit light of all wavelengths less than 640 nm and reflect all wavelengths greater than 640 nm to the side. In contrast, a 500 nm long-pass dichroic mirror (500 LP) will transmit all light of wavelengths greater than 500 nm, but reflect to the side all light less than 500 nm. (These dichroic mirrors are angle sensitive; they are usually used at 45° to the light path. If the angle is changed, the wavelengths of the transmitted and reflected light will also change.)

By following the light path in Figure 5.7, we can see the way dichroic mirrors (in an example of a typical two-scatter parameter plus three-fluorescence parameter system) can be used to partition multicolor light. The first dichroic mirror (a 500 nm LP) reflects all light less than 500 nm to the side toward one photomultiplier tube (PMT); all light with wavelengths greater than 500 nm is transmitted straight ahead. At the next dichroic mirror, a further partition takes place: All light with wavelengths greater than 640 nm is reflected to the side from this 640 nm SP mirror, and light less than 640 nm is transmitted. Because light less than 500 nm has already been removed from this beam, the transmitted light at this stage will be of wavelengths between 500 and 640 nm. At the final dichroic mirror in this system, light greater than 560 nm will be reflected out of the beam (because of the previous step, this light will be between 560 and 640 nm), and all light shorter than 560 nm will continue straight ahead (being between 500 and 560 nm because of previous partition steps). By use of a system such as this:

- Side scatter light (488 nm) will be reflected out of the beam path toward the first PMT by the 500 LP mirror
- Far-red fluorescence will be reflected out of the beam path toward the second PMT by the 640 SP mirror
- Orange fluorescence will be reflected out of the beam path toward the third PMT by the final 560 SP mirror
- Green fluorescence will be transmitted straight ahead through that final mirror toward the last PMT in the optical system

By the use of filters with particular color specifications immediately in front of the photodetectors, we have our last chance for making sure that the only light registered on a given photodetector has been derived from a specific fluorescence color. Filters are similar to dichroic mirrors, but are meant to be used at 90° to the light path; they transmit light according to its color. Filters come in three basic types: A short-pass filter will transmit all light of a wavelength less than the specified value; a long-pass filter will transmit all light of a wavelength greater than the specified value; and a band-pass filter will transmit only light in a narrow wavelength band around the specified value. Band-pass filters are of most use in flow systems because they are most specific in their transmission characteristics. These characteristics are usually described according to the peak transmission wavelength and the width of the wavelength band at 50% of that peak transmission. For example, if a filter transmits 90% of the 570 nm light falling onto its surface, and if this percentage of transmission drops to 45% when the wavelength changes by 15 nm in either direction, then the filter will be described as a 570/30 nm band-pass filter.

Different filter and mirror combinations will be required for experiments using different combinations of stains. By looking at the detailed fluorescence emission spectra of the fluorochromes in use, we can plan a strategy for selecting the filter and mirror sets that will allow us to correlate the signal from a given photodetector with the fluorescence from a given fluorochrome. Figure 5.8 shows the fluorescence emission spectra from fluorescein and phycoerythrin drawn on the same axes. (These two fluorochromes are the most useful example for our purposes here, but the same principles and filter strategy would apply to any group of two or more fluorochromes used simultaneously.) By inspection of these spectra, we can see that the best dichroic mirror to use for splitting fluorescein fluorescence from phycoerythrin fluorescence is a 560 nm mirror. This will send most of

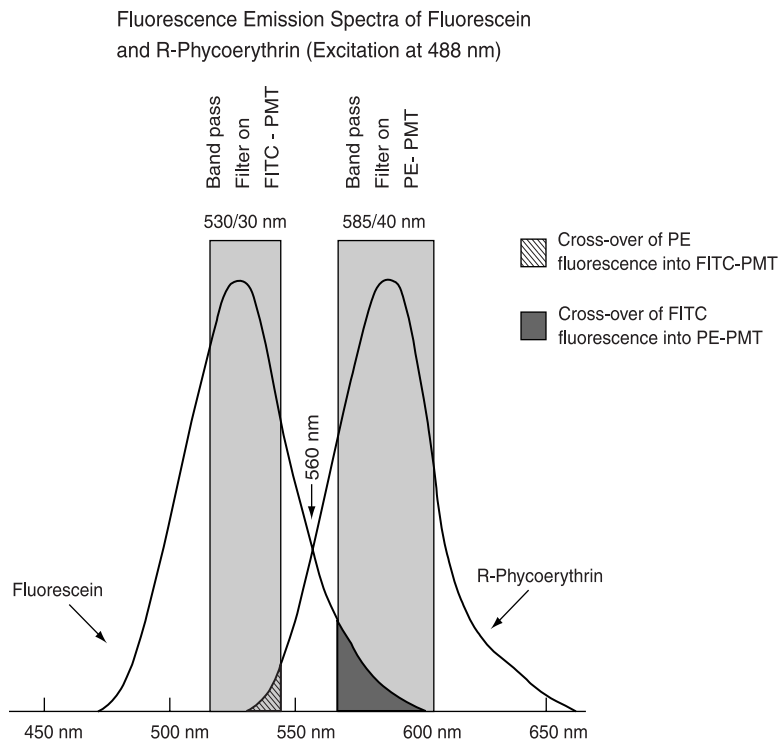


Fig. 5.8. The cross-over of fluorescein and phycoerythrin fluorescence through the filters on the “wrong” photodetectors.

the fluorescein fluorescence straight through to one photodetector and reflect most of the phycoerythrin fluorescence toward a different photodetector. By fitting the green detector with a 530/30 nm filter and the orange detector with a 585/40 nm filter, we have constructed a system that will register fluorescein fluorescence and phycoerythrin fluorescence mainly on different photodetectors. We might be entitled now to call one photodetector the “fluorescein detector” and the other one the “phycoerythrin detector”; but that qualifying word *mainly* lies at the root of our next problem.

SPECTRAL COMPENSATION

With the filter and mirror combinations just described, a cell stained only with fluorescein will emit fluorescence that will register strongly on the green-specific photodetector (and this is fine if we are using

only fluorescein as the stain in our system). However, inspection of the fluorescence emission spectrum of fluorescein (Fig. 5.8) indicates that some of the light emitted by a cell or other particle stained only with fluorescein will manage to evade our strategic obstacles (mirrors and filters); some of the light emitted by fluorescein is of a wavelength long enough to register on the orange photodetector. This is another way of saying that the light emitted by fluorescein is “orange-ish green.” No matter how tightly we restrict the filter and mirror specifications, we cannot get round the fact that there is a significant degree of overlap between the fluorescence emission of fluorescein and that of phycoerythrin. Therefore, there is no way to keep all the fluorescein-derived light from the orange detector without losing most of the phycoerythrin fluorescence as well. If we want to measure PE fluorescence with reasonable sensitivity, then the orange photodetector will also respond to a limited extent to fluorescein fluorescence. Our use of filters and mirrors must, for these reasons, be a compromise.

What this compromise means, in practice, is that a cell stained only with fluorescein will register a signal brightly on the green-specific photodetector but also significantly on the orange-specific photodetector. Similarly, a cell stained only with phycoerythrin will register a signal brightly on the orange detector, but also slightly on the green detector. The left panel of Figure 5.9 shows how these pure signals look on dot plots giving the relative intensities for the signals on the orange and green PMTs. Clearly, we would have been incorrect in calling the detectors fluorescein or phycoerythrin specific; the filters and mirrors in front of our photodetectors cannot make them as specific as we would like them to be. The correct terminology should, therefore, simply identify a photodetector by the specifications of its filter (that is, a “530/30 nm detector” or a “585/40 nm detector”). When flow cytometrists, being as lazy as everyone else, talk about a “fluorescein photodetector,” you now have enough knowledge to understand the imprecision of this phrase. In many cytometers, the fluorescence detectors are simply given numbers (e.g., FL1, FL2, FL3). If the filters are fixed (as in some benchtop instruments), the user needs to refer to the technical manual to know which filters are in front of which detectors. In a research cytometer, the filters can be changed to adapt to the use of different combinations of fluorochromes; knowledge of the exact optical pathway in place at any one time becomes correspondingly much more important.

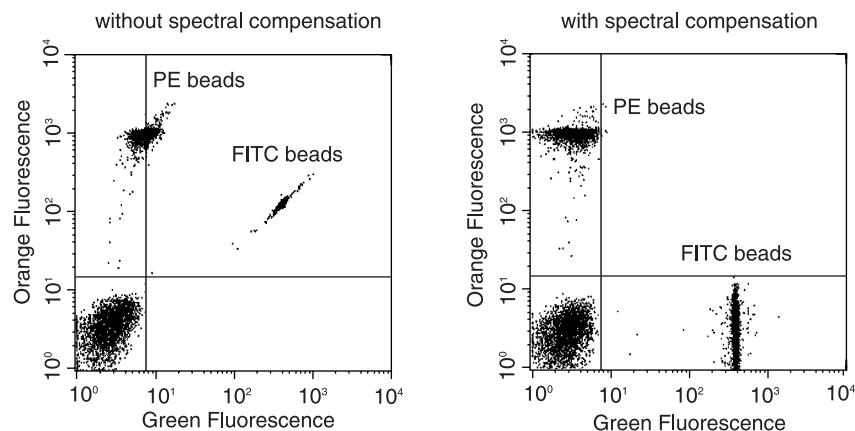


Fig. 5.9. The fluorescence from phycoerythrin beads registers a bit on the green photodetector; and the fluorescence from fluorescein beads registers considerably on the orange photodetector. Compensation (right) allows us to correct for this cross-over.

Having understood why there is cross-over or overlap between fluorochromes, each registering to a certain extent on the “wrong” photodetector, we now must decide what to do about it. What we do about it is called *compensation*. Cytometers are provided with an electronic circuit, a compensation network, that measures the intensity of the signal on one photodetector and subtracts a certain percentage of that signal from the signal on another photodetector (because a certain fixed percentage of any fluorescein signal will always cross over and register on the 585/40 nm phototube, and a certain fixed percentage of any phycoerythrin signal will always cross over and register on the 530/30 nm phototube).

Those percentages are routinely determined empirically. Cells brightly stained with only fluorescein are run through the cytometer; their signals on both the green and orange photodetectors are recorded; and the percentage subtraction (percentage of the signal on the green detector to be subtracted from any signal on the orange detector) is varied until no signal above background is registered on the orange photodetector. Technically, we want to make sure that the median orange channel of fluorescein-stained cells is identical to the median orange channel of unstained cells. We then go through the same procedure with cells that have been brightly stained only with phycoerythrin in order to determine what percentage subtraction

is required to compensate the green photodetector for cross-over from the phycoerythrin fluorescence. When both photodetectors have been correctly compensated for cross-over from the wrong fluorochromes, our signals, plotted as two-dimensional dot plots, should look like those in the right panel of Figure 5.9.

If more than two fluorochromes have been used for simultaneous multiparameter analysis, then the signals from all PMTs need to be compensated individually in the same way with respect to each fluorochrome (i.e., in a three-color example, the green PMT needs to be compensated for cross-over fluorescence from both the red and orange fluorochromes; the orange PMT for cross-over fluorescence from the green and red fluorochromes; and the red PMT for cross-over fluorescence from the green and orange fluorochromes). In practice, this involves staining cells separately and brightly with each of the three fluorochromes. The separately stained cells can then each be mixed with unstained cells; the three mixtures can be run through the cytometer and examined with respect to three different dot plots (red vs. green; green vs. orange; and red vs. orange) to make sure that the compensation network gives square patterns (as in Fig. 5.9, right panel) for the cells in all three plots.

As a general comment, compensation is one of the more difficult areas in flow cytometry and gets more difficult as more colors are involved. For this reason, there is software available that permits postexperiment compensation: Samples can be run through the cytometer without any or with minimal electronic compensation, and the compensation subtraction can be applied or adjusted afterward during data analysis. There are great advantages to software compensation because, especially in multicolor experiments, it is often difficult to “get it right” while under the pressure of acquiring data at the cytometer bench. Of course, even postexperiment compensation still requires that files from single-stained samples be stored so that the software compensation subtraction can be evaluated.

It remains to be said (unfortunately) that the compensation values are valid only for a particular pair of fluorochromes with a particular set of filters and mirrors and with particular voltages applied to a particular set of PMTs. If any one of those elements is altered, the required compensation values will alter as well. In general, once compensation has been set using single-stained particles with a given experimental protocol (PMT voltages, filters, and so forth), compensation values between a given pair of fluorochromes should remain

constant within that protocol from day to day. However, because compensation can affect the way two-color plots are evaluated, correct compensation can be of critical importance in the interpretation of data from samples that are brightly stained with one fluorochrome but weakly stained with another. For this reason, single-stained controls should always be run within each experimental group in applications where this type of interpretation is required.

Through the chapters so far, a basic cytometric system has been described. We have generated an illuminating beam, followed the fluid controls that send cells through the center of that beam, and registered the light signals emerging from those cells as specifically as possible on individual photodetectors, according to their color. We have also compensated for any lack of specificity in our photodetectors that might result from spectral cross-over between fluorochromes. Finally, we have stored the information from the light signals from each cell in a computer storage system so that they can be analyzed in relation to each other through computer software. We are now in a position to begin to look at ways in which such a flow system might be used. In the following chapters, applications will be described to give some indication of the common, the varied, and the imaginative practices of flow cytometry. My choice of specific examples is intended to illustrate points that may be of general importance and also to illustrate a range of applications that might stimulate interest in readers who are new to the field. It is not meant, nor in a short book could it hope, to be exhaustive.

FURTHER READING

Chapter 2 in **Ormerod** is a good, gentle discussion of fluorescence technology and light detection. Chapter 4 in **Shapiro** is slightly more detailed, and Chapter 2 in **Van Dilla et al.** follows well from there. The **Melles-Griot** catalog is a valuable (and free) source of information about lasers and filters.

Chapter 12 in **Melamed et al.**, Section 4 in **Current Protocols in Cytometry**, and Chapter 7 in **Shapiro** discuss the uses of different fluorochromes. With respect to the physical characteristics of these fluorochromes, the **Molecular Probes Handbook** is a valuable (and free) source of information. Chapter 1.14 in **Current Protocols in Cytometry** is a detailed discussion by Mario Roederer of compensation.